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PRECLINICAL EXPERIMENTAL SCREENING MODELS FOR THE ASSESSMENT OF ANTI-CANCER DRUGS

Chandrashekhar Singh, Shaheen Khan, Falak Bamne and Imtiyaz Ansari *

Department of Pharmacology, Oriental College of Pharmacy, Navi Mumbai - 400705, Maharashtra, India.

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Correspondence to Author:
Mr. Imtiyaz Ansari

Assistant Professor,
Department of Pharmacology,
Oriental College of Pharmacy,
Navi Mumbai - 400705, Maharashtra, India.

E-mail: imtiyaz.ocp@gmail.com

ABSTRACT: Cancer, according to WHO, is a generic terminology for a group of diseases that are characterized by abnormal growth of cells. This uncontrolled, abnormal growth is beyond the natural boundaries of normal cell growth, which can then metastasize to adjoining or distant sites of the body and/or to the organs. Cancer is the second most leading cause of mortality and has resulted in 9.8 million deaths in 2018 alone. Efforts to explore newer anticancer drugs have significantly increased in recent years. Appropriate preclinical experimental screening models can help us apprehend the pathogenesis, complications, and testing of various therapeutic agents. Both *in-vitro*, as well as in-vivo models are available for the screening of compounds. A preclinical screening model should have a high sensitivity and reproducibility. This review summarizes some conventional as well as newer experimental screening models used for preclinical screening of test compounds for the treatment of cancer.

INTRODUCTION: WHO defines cancer as a "Generic terminology for a group of diseases that are characterized by abnormal proliferation of cells." This abnormal proliferation of cells can metastasize to adjoining tissues and remote organs. Various types of models are summarized in this overview and it is important to keep in consideration that these experimental screening models do not directly 100% correlate to human cancer, and the outcome obtained from performing studies on these models may or may not be active in a similar manner on humans in clinical trials. Also, cancer progression has much redundant. Survival, as well as growth regulatory pathways in the total course of their progression, makes it very adaptive in nature and difficult to target. This dynamic and variable nature of human cancer and the variation in the rate of progression of cancer in animal models when compared to humans must be considered during the evaluation of the observations of these experimental models in order to correlate with human cancer 1-5 properly. Preclinical, experimental screening models can help us discover new therapeutic agents, new therapeutic agents in combination with standard approved [1, 6-10].

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However, conventional experimental screening models have an attrition rate of 95%, *i.e.*, the success rate of 1 in 20 for oncology drugs, and are also time-consuming. Such attrition rates are because of limited strengths, efficiency, and predictive capability of various preclinical screening designs and models as older and outdated

models and designs have led to higher attrition rates. Newer and efficient, and robust experimental models are therefore required to help screen the cancer drugs efficiently and with relative ease [1, 11-14].

***In-vivo* Models:**

1. Carcinogen Induced Tumour Models: Chemical carcinogenic model (Also known as Autochthonous Tumour Model) in preclinical screening is one of the most widely used and oldest models used in cancer screening. This model has a high correlation and high translation in clinical trials and also helps us explain the multistep carcinogenesis and the mechanism of tumour development [1, 15, 16]. It is already established from a long time that cancer can be caused because of exposure to an environment that is rich in toxic chemicals. Such chemicals can cause DNA damage and initiate mutagenic modifications and ultimately uncontrolled growth, *i.e.,* cancer. DNA sequencing allows us to inspect a tumour that contains high point mutations, and these mutations signatures reflect the causative agent that was used for inducing the tumour. This principle became a route to identify the cause of mutation in human cancer cells [15]. Yamagiwa and Ichikawa induced tumour on rabbit skin using coal tar in 1918, and then after many trials of experiments, cancer was induced on mice for evaluating the NCE for associating the anticancer property of the test agent with the reduction in the size of the tumour. Tumour development is a multistage process *i.e.,* separate stages of initiation as well as progression in a time-dependent manner upon the action of various carcinogens [1, 15]. A single initial exposure to a mutagenic/carcinogenic chemical can lead to the initiation of a tumour but the progression is more likely to occur after multiple exposures to other agents known as promoters. Promoters act by stimulating inflammatory response and proliferation rather than DNA damage [15, 16].

The general mechanism by which carcinogens cause cancer is that they form adducts with the DNA base pairs and cause irreversible damage, because of which mutations occur [15, 17]. Some of the most commonly used carcinogens are DMBA (7,12-dimethyl-benz(a) anthracene), AOM (Azoxy-methane), NMU (N-nitroso-N-methylurea), MNNG (N-methyl-N-nitro-N-nitrosoguanidine), and even some tobacco-smoke related carcinogens and nitro-samines like DEN (diethyl-nitrosamine), NNK (4-methyl – nitrosamino - 3 - pyridyl - 1 - butanone), Bap (benzo (a) pyrene). These carcinogens are either used alone or along with a tumour promoter agent such as phorbol esters to induce a specific type of desired cancer. Mainly immune com-promised mice are used and the susceptibility to induce cancer depends upon the dosage, carcinogen itself and the strain used. Some commonly used carcinogens along with the type of cancer they can induce are listed in **Table 1.**

TABLE 1: CARCINOGENS ALONG WITH THE TYPE OF CANCER THEY INDUCE [18-27]

Carcinogen	Type of Cancer Induced
Nmu	Mammary carcinoma
Den	Hepatocellular carcinoma
NMU and MNNG	Gastric carcinoma
Bop	Pancreatic ductal carcinoma
Aom	Colorectal carcinoma
Nnk	Lung carcinoma
DMBA Andbap	Squalors cell carcinoma (SCC) models of the skin and upper aero digestive tract in rats, mice and the hamster oral mucosa

Carcinogen induced tumour model has some distinct advantages regarding clinical relevance. The carcinogens can induce lesions that are reproducible and organ-specific. Also, they have a high correlation with human cancer because of the molecular, histopathological, and biochemical similarities, and also the tumor-induced is capable of metastasizing to other distal organ sites [20-22, 25-28]. However, this model does not identify the therapeutic efficacy of a therapeutic agent for a specific stage of cancer. Unlike in the case of GEMM, xenograft model or hollow fiber tube model, which relies on imaging technology to quantitatively assess the therapeutic efficacy of the test agent on the metastatic progression and tumour growth, the carcinogen-induced tumour growth and metastatic progression can be easily accessed by electronic callipers [1, 2, 19-25, 29].

Apart from the merits of the carcinogen-induced tumour model, it has some inherent limitations and flaws as well. The major setback is the long duration of time it requires for the carcinogen to introduce a tumour and lack of immune system. A carcinogen can take from a minimum of 4-5 weeks up to even 50-60 weeks. The time for the induction

of a tumour depends mainly upon the carcinogen being used, provided that the dose, dose-frequency, dose-timing, rodent strain used is optimized. Carcinogens with their respective estimated time period required for induction of tumour is listed in **Table 2.** Another demerit being the safety concern because of the long-term handling of carcinogens and promoters. Also, not to forget the associated animal maintenance cost and extra care as immune-compromised strains are being used.

TABLE 2: CARCINOGENS WITH THEIR RESPECTIVE ESTIMATE TIME PERIOD REQUIRED FOR INDUCTION OF TUMOR [18-25]

Carcinogen	Estimate time Required for Induction of Tumour
NMU	5-6 weeks
AOM	40-50 weeks
BOP and other nitrosamines	24-50 weeks
NNK	24-50 weeks
DEN	24-50 weeks
HCC	24-50 weeks

Despite the disadvantages or limitations, the model is still widely used because of the molecular, histopathological, phenotypical, and biochemical similarities to that of human cancer. It also has the capability to explain the pathological stages of tumour development *via* separated stages of initiation and progression in a time-dependent manner.

2. Xenograft Models with Established Tumour Lines: Tumour cell lines or tissues can be from the same or different species of mice/rat, or various human cancer cell lines can be transplanted in mice/rat that is immune-compromised, which leads to tumour induction. Tumour cell lines that are cultured *in-vivo* are implanted into the mice, which are immunodeficient.

A normal mouse's adaptive immune system and humoral immune system will induce an immunogenic response in order to cause apoptosis of the implanted tissue/cells which would result in rejection, and that will be of no use if the tumour has to be induced. But on the other hand, tumour induction is much more effective in immune-compromised mice because the implanted tissue/cells are not rejected easily [10, 30-32]. The evaluation of the potential candidate is based on the change in the size of the tumour after the introduction of the evaluation compound in the tumor-induced mice.

Based on the region of transplant, whether it is analogous to the original region in the body or not, the xenograft model is of two types: a) Ectopic xenograft model, b) Orthotopic xenograft model[1].

Ectopic Xenograft Model: In this model the tissue/cell is implanted on a non-analogous region from which it was obtained. Most commonly, via a subcutaneous injection which allows the tumour to develop on the surface of the mice. This is a standard model of choice when it comes to validation and assessment in oncology studies. The most prominent advantage of ectopic xenograft model is it makes the visual observation and quantification of the tumour development on the surface of the mice/rat over time relatively easy. Also, it doesn't require any advanced surgical skills and, therefore, consumes less time and effort. On the other hand, as immune-deficient mice are being used, the correlation is less as the immune system is not taken into account and therefore is not suitable for those compounds that modulate the immune system as a mechanism. Also, because of the non-analogous transplantation, the tumour growth (metastasis) characteristics are different from the analogous origin of tumour [10, 31-34].

Orthotopic Xenograft Model: It allows for the tumour tissue/cell to develop in the region which is analogous to the origin of the primary tumour. The clearest advantage of this model is the high correlation, as the tumour development here is taking place in the analogous region, and therefore, the characteristics will most likely be the same as the primary tumour development. Even metastatic characteristics are similar sometimes if not always and can be studied.

The major disadvantage is that the visual observation and tracking of tumour growth or metastasis is very complicated and most often requires a sacrifice of the animal. Expensive and complicated techniques are also an option if sacrificing is not feasible like using cancer cell lines with express markers (fluorescence or luciferase) which then can be observed using optical imaging, computerized tomography (CT) or magnetic resonance imaging (MRI). Other disadvantages include the requirement of well-trained experts with surgical skills for the surgeries for transplanting the tumour tissue/cell to the

orthotopic region. Also, the endpoint for determining the effects of therapy are very complex and most often, survival is the only feasible and practical endpoint [10, 31-34]. Some examples of cancer cell lines used in the xenograft model are listed in **Table 3.**

TABLE 3: COMMONLY USED HUMAN TUMOR CELL LINES IN XENOGRAFT MODELS [31-34]

Type of Tumor Origin	Good Cell Culture Lines	Acceptable Cell Culture Lines
Colon	KM12, SW-620, HCT-15, HCT-116	HT29, DLD-1, HCC-2998, COLO 205, KM20L2
CNS	U251, SNB-75, SF-295	-
Lung (Non-Small Cell)	NCI-H23, NCI-H522, NCI-H460	EKVX, NCI-H322M, HOP-92
Mammary	MX-1, ZR-75-1	MDA-N, UISO-BCA-A, MCF-7, MCF-7/ADR-res, MDA-MB-231, MDA-MB-435
Ovarian	SK-OV-3, OVCAR-5	IGROV1, OVCAR-4, OVCAR-3
Melanoma	SK-MEL-28, LOX-IMVI.	M14, UACC-257, SK-MEL-5
Prostate	PC-3	Du-145
Renal	RXF393, CAKI-1	SN12C, RXF631, A498

3. Hollow Fibre Assay (HFA): Currently, the most widely used models to perform *in-vivo* anticancer drug screening are xenograft model and hollow fibre tube assay. However, HFA is not a standalone model to be used and is just a substitute for a secondary model. The xenograft model still is widely used because of its high correlation, but it has its own disadvantages and limitations, and above all it is costly and time-consuming to perform. Therefore, to streamline the process, HFA as a pilot is developed to be performed prior to the xenograft model to save resources. Compounds that screen HFA is then a candidate for xenograft model [35, 36].

The fundamental outcome of the model is the same as the xenograft model, *i.e.*, HFA also measures the activity of anticancer drugs against cancer-induced animals *via* various cancer cell lines on immune-deficient mice, just the method of induction and site of induction are the variables that have changed. The method of induction and site of induction in HFA is what sets it apart and makes it very reproducible, efficient, and easy to observe and track overtime. It cannot be used as a standalone model because it has a very low correlation and does not simulates an actual biological environment of cancer growth and therefore depends on the xenograft model for the final validation [35, 37]. The cell lines used for HFA screening are listed in **Table 4.**

TABLE 4: COMMONLY USED HUMAN TUMOR CELL LINES IN HOLLOW FIBRE ASSAY [35]

Tumour Origin	Cell Lines
Non-Small Cell Lung cancer	NCI-H23, NCI-H522
Breast Cancer	MDA-MB-231
Colon Cancer	SW-620, COLO 205
Ovarian Cancer	OVCAR-3. OVCAR-5
Melanoma	MDA-MB-435, LOX, UACC-62

HFA is based on tumour cell's ability to form a tumour in hollow tubes consisting of PVDF (Polyvinylidene fluoride). The inner layer of the hollow tubes is connected to a layer of living cells that are in contact with necrotic cells. Tumour cell lines are initially cultured until they reach a stage, the log phase growth stage, after which the cells are introduced inside hollow fiber tubes (PVDF) 1 mm diameter and 2 cm length. These hollow fiber tubes are then incubated for a duration of 24 to 48 h.

PVDA are semi-permeable and, therefore, allow nutrition and potential anticancer drugs to pass through them. After about 24-48 h, the tumour cell line incubated hollow fiber tubes are implanted either subcutaneously or *via* intraperitoneal route into an immune-deficient mice strain (Note that each animal can receive up to 3 implants in order to reduce the usage of animals) [38].

After about 3-4 days, the test drug is administered via I.P. for the next 4 more days while the implant is still inside mice. On the 5^{th} or 6^{th} day, the tubes are removed, and the cell viability assays are performed like MTT Assay to count the viable cells and compare the control, test, and standard groups, and therefore, the percent inhibition can be determined. Cell cycle analysis, induction of apoptosis can also be determined [39].

Similarly, the drug is tested for subcutaneous injection and after it passes both I. P and S. C. the drug is then a candidate for the xenograft model. One more disadvantage of HFA model is the

limitation of space for the tumour to grow, and thus, the tumour growth is limited by the diameter of the hollow tube. Another drawback is the walls of the hollow fiber tube only allow permeation of particles up to 500 kDa and, therefore, some antibodies and essential nutrients larger than 500 kDa, which makes the correlation even less [40].

4. Genetically Engineered Mouse Models (GEMM): In contrast to using immune-deficient mice, which does not take the immune system into consideration, tumours can be directly induced on mice by genetically modifying them. These genetically engineered mice are called GEM *i.e.*, Genetically Engineered Mice. In GEMM, instead of modulating the immune system and then introducing cancer, mice themselves are genetically modified to grow tumour on their own. The major advantage of this model is that the immune system is an added variable that can affect the experiment results, which makes the result more validated and acceptable and thus increases correlation [8, 35, 36] for the development of anticancer drugs other than.

There are several methods of transgenic modification of a mouse, each for a different type of cancer induction. (Not only mice, but this model is also relevant for other organisms like zebrafish, drosophila). It is possible via DNA sequencing technologies like CRISPR to perform transgene delivery of specific oncogene of interest or a promoter into the DNA construct of the isolated host cell and then microinject the modified DNA construct into the mice.

This introduction of an oncogene or "Knock in" of an oncogene is for the overexpression of that gene via the host cell's promoter and enhancer region. This process is known as "knock-in gene" process. On the other hand, any specific gene (mainly tumour suppressor genes) can also be removed or "knocked off" in order to reduce the expression of that specific gene. This is known as "knock off gene" process or gene silencing. [41].

Knocking-in or off any specific gene will cause overexpression or downregulation of that gene, respectively, which will be responsible for the induction of tumour related to that gene[42]. The modified DNA construct can also contain reporter genes like GFP, RFP, Luciferase.

This will enable the tracking or observation of the tumour development, *i.e.*, metastasis, without sacrificing the mice. Examples of genes that are knocked in for overexpression of certain enzymes that can induce cancer are PI3K genes which are responsible for the expression of enzymes like PIK3CA, PIK3CB, and PIK3CD. Oncogenes include TSC gene, Cre-lox (Note that these genes are not technically inherently oncogene, but their overexpression can induce cancer.) In order to induce cancer in a specific region/tissue, the regulatory genes of those regions/tissues can be used.

For example, the gene which is responsible for the expression of long terminal repetition of mouse mammary tissues can be used to induce breast tumour, which is similar to human breast tumour [43]. On the other hand, certain tumour suppressor genes like PTEN, Rb gene which is responsible for the regulation and to prevent overexpression of oncogenes. Thus, by knocking off such genes, cancer can be induced. For instance, the model for non-small cell lung carcinoma is conducted by simultaneous activation (overexpression) of the K-RAS gene and deactivation (gene silencing) of Rb and p53 [8, 35].

The offspring produced by these genetically modified animals also express the same modifications, and therefore, the modifications can be monitored even in very early stages of development, for example, embryonic stage [35]. By selectively modifying genes, researchers have already developed mouse models of lung, breast, colon, ovary, pancreas, and prostate cancer [35].

The only major limitation of GEMM is the level of complication involved, technical skills required, and the cost. Also, it requires the use of expensive and complicated photosensitive detection systems for the observation of tumours. Genetically modified mice also have a tendency to produce spontaneous and multifocal tumours [35, 36].

Animal's life span and the volume of tumour is not the only deciding factor in considering the efficacy of a potential anticancer agent, it is even sometimes not reliable as a tumour can even shrink in volume may have spread to other organs of the body. In such cases, sacrifice of mice is the only viable

option; GEMM helps solves this problem as it allows visualizing the tumour growth using reporter genes even in early embryonic development stages [44].

***In-vitro* Models:** Various approaches *in-vivo* methods have emerged, which rely mainly on cell-based screening to evaluate potential anticancer drugs for their efficacy. *An in-vitro* assay is a mechanism-based approach, *i.e.*, it targets the molecular malignancy, which is either established or proposed to be the reason for cancer [45, 46].

In-vitro models can be performed on a small scale but preferably on a commercial scale; it is performed *via* HTS *i.e.*, High Throughput Screening. HTS is a drug discovery process and a miniaturized *in-vitro* assay format that employs a group of techniques to conduct assay of a large number of combinatorial libraries for their ability to modify the properties of a specific biological target and or pathway for further validation in additional biological and pharmacological experiments. In other words, HTS is a process of screening a large number of compounds against a number of targets per unit time, which generates more hits in less time and then subsequently generating more products in less time. HTS can screen up to 100,000 compounds in a day, and above 100,000 is called uHTS (ultra HTS) [47, 48].

In-vitro models are either cell-based assays or biochemical assays. Cell-based assays are types of *in-vitro* assay techniques in which the test compounds are tested against living cells (cell population or single cell). The whole cell-based assay is used when the exact steps involved in the mechanism of disease or target is not well defined so rather than using the exact parts on which the drug will act, whole-cell is used. Examples of responses from a living cell can be cell viability, cell death (%inhibition), motility, proliferation, toxicity, and change in morphology (*e.g.*, Cell wall disruption). Depending upon the response/property which is being required on the living cell upon the action of the drug, there are different types of cell-based assays are A) Phenotypic assays. This is to evaluate cellular processes like cell motility, cell proliferation, cytokinensis, and cell viability. B) Second messenger assay. This assay evaluates the second messenger mobilization in various cellular pathways. C) Reporter Gene Assays For evaluation of transcriptional and gene expression activity. Biochemical assays use biochemical targets isolated from the cell rather than using the whole cell. While cell-based assays are more biologically relevant, biochemical assays are easier to perform. Biochemical assays are also known as mechanism-based assays and are cell-free *in-vitro* assays that model the biochemistry of individual targets inside the cells. Biochemical assays are based on the interaction of the test compound with the biochemical targets isolated from the cell, such as an enzyme, hormone, and receptor [47-51].

The goal of using molecular targets or cells is to improve the selectivity and efficacy of cancer treatment [45]. Also, compared to the animal models, *in-vitro* methods are less time-consuming and relatively less expensive; therefore, it allows screening of a larger number of test compounds in less time. However, the assay is being performed at a very miniaturized scale which is not always reliable, and thus validation from higher-level animal models is required. However, it plays a major role in screening a large number of drugs and narrowing down the number of drugs in the drug development process [45].

1. Enzymatic Assays:

1.1. Tetrazolium Salt Assays: Tetrazolium salts can undergo reduction under the presence of mitochondrial enzymes, which are mainly present in the cytosol of the cell and leads to the formation of a coloured compound called formazan. Since the presence of these mitochondrial enzymes is an indication of a viable cell because the viable cell will have metabolic activity due to NAD (P) H flux action.

Non-viable cells or cells with low metabolism are not incapable of reducing the salts. The amount of colour change is, therefore, an indication of cell viability and can be used for cell viability assays.

There are a number of tetrazolium salt available which are used for cell viability assays like MTT, XTT, MTS, WSTS. Such salt-based assays are time-dependent, therefore, incubation time should be limited and tightly controlled. Also, these salts are sensitive to light, and therefore, these assays are performed in dark conditions [45, 52, 53].

MTT Assay: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a tetrazole which is yellow in colour. After it reacts with the mitochondrial enzymes, it is converted into a purple coloured formazan compound. A solubilization solvent is added in order to make the insoluble formazan soluble. The amount of purple coloured formazan product is quantified using a spectro-photometer at 500 to 600 nm. The lower the absorption, the higher the presence of formazan, which is an indirect quantification for the cell viability [45, 52, 53].

XTT Assay: XTT (2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) another tetrazolium salt is also used in a cell-viability assay based on the same principle of MTT assay. It is supposed to overcome certain limitations of MTT Assay and is being slowly replacing the MTT Assay. XTT Assay is more sensitive and accurate, and the product obtained is already water-soluble; therefore, the use of solubilization solvents is reduced [45, 54, 55].

MTS Assay: MTS (3-(4, 5-dimethylthiazol-2-yl)-5 - (3 - carboxymethoxyphenyl) - 2 - (4 - sulfophenyl) - 2H - tetrazolium) which is yet another tetrazolium salt which under the presence of PMS (Phenazine methosulfate) produces the coloured formazan product. This formazan can be quantified at an absorbance maximum of 490 nm in the presence of phosphate-buffered saline. MTS has the advantage that reagents can be directly added to the cell culture by skipping the intermediate steps, which have to be done in an MTT Assay. Therefore, it is also known as "one step MTT". Direct addition of reagents, however, makes this assay susceptible to interference in the colorimetric quantification. However, MTS assay is essential which is used as a confirmatory assay after either MTT, XTT or any other assay [45, 56, 57].

1.2. Sulforhodamine B (SRB) Assay: SRB is a pink-coloured aminoxanthine dye that is anionic in nature and is capable of binding to the basic amino acid group residues of a cell culture under moderately acidic conditions [58, 59]. The acidic condition is maintained using 10% (wt/vol) trichloroacetic acid. Based on the cellular protein content present in the cell culture, the cell culture takes up as much dye as required for the reaction, and after 30 min of the incubation period, the leftover dye is washed off using 1% (vol/vol) acetic acid. The absorbance is then measured at a wavelength ranging from 560 and 580 nm. Alternatively, if HTS is being done then microtiter plate readers are used on a 96 well format. The binding (staining) of the SRB dye is stoichiometric in nature; the amount of dye that is obtained from cell culture after incubation is directly proportional to the cell mass [58, 59]. Quantification of cell viability, cell density as well as cytotoxicity can be done using SRB assay [59]. Tetrazolium salt assays, although are adequate for reliable quantification of viable cells, there are many variables that, if didn't go right, can lead to altered observation. For example, if the duration of incubation with reagents and cell culture is not proper may lead to less formation of formazan, or the reagents themselves are toxic to some extent and can result in cytotoxicity which may be expressed as false overactivity of the test compound. To overcome such limitations, the next majorly used assay is SRB *i.e.,* Sulforhodamine B (SRB) assay [45, 58-60].

Membrane Integrity Assays:

2.1. Tryptan Blue Exclusion Dye Test: Tryptan blue exclusion test is a very straightforward and primitive assay to determine cell viability and cytotoxicity in cell culture. Tryptan blue is a dye that is capable of permeating into non-intact cell membranes and binding to the intracellular proteins and rendering the colour of the cell to blue [45]. This assay is based on the principle that every viable cell that is intact in nature will have a well-maintained cell membrane and which will prevent the inner cellular structures and cytoplasm from dyeing whereas, dead cells do not have intact cell membranes, and the inner cellular structures are exposed to dye like tryptan blue, eosin.

When a cell-culture is mixed with dyes like tryptan blue and kept for incubation, live cells will exclude the dye, and only dead cells and their cytoplasm will get dyed. These wills indicate the count for viable cells. Cell viability can be determined prior to incubation with the test drug and after to determine the efficacy of the drug [61].

2.2. LDH Assay: LDH *i.e.,* Lactate dehydrogenase, is an enzyme that is present in the cytoplasm of eukaryotic cells.

This enzyme is released from the cell after cell death occurs and the cell membrane is disrupted. Various anti-cancer compounds are capable of disrupting the integrity of these cellular membranes. The cell culture is incubated along with lactate, NAD^+; upon contact with LDH if any dead cells are present, the LDH will reduce the NAD^+ to NADH. The presence of NADH can be measured using Tetrazolium salt assay using the tetrazolium salt INT. NADH will reduce the INT salt to red-coloured formazan product. This formazan product can be quantified at a wavelength of 490 nm spectrophotometrically. The amount of coloured formazan formed is proportional to the of cells lysed [45, 62-64].

2.3 H-Thymidine Incorporation Assay: 3H-Thymidine is a nucleoside which is radioactive *i.e.* radiolabel led which is used in this assay to evaluate cell viability, cell proliferation, cytotoxicity. Incorporation of 3H-Thymidine is made into the chromosomal DNA strands of the cell culture. After the incorporation of 3H-thymidine, which is radioactive, if the cells undergo proliferation, the new cells formed will also have the same modified DNA, which will contain the 3H-thymidine, which can be easily observed using a scintillation beta-counter. The data obtained gives a direct quantification about the extent of cell division that has taken place [45, 65].

Non-conventional Newer Models:

1. Anti-angiogenic Models: Angiogenesis is a physiological multistep process by which the formation of blood vessels occurs from a new site or from pre-existing vasculature. Angiogenesis is a normal physiological process that is required for the formation of new blood vessels especially during the embryonic development stage, wound healing, inflammation. However, tumour development and metastasis during cancer are known to depend on angiogenesis which serves as a feeding or nurturing mechanism for the tumour cells [66, 67]. Thus, targeting angiogenesis could prove a very efficient strategy to control tumour progression and metastasis [68].

1.1 Zebrafish: Caudal Fin Regeneration Model: Zebrafish (Danio rerio) is a fish found in freshwater belonging to the family minnow. It is a tropical fish [69, 70].

It is an important and relatively newer vertebrate model organism in drug development. Zebrafish has many advantages that set itself apart from the conventional *in-vivo* models. For example, it has regenerative abilities, the embryo is transparent and develops outside their mother, and therefore, it is easy to study angiogenesis in real-time during the embryonic development phase, the genome is already completely sequenced, and the DNA makeup and general anatomy of zebra fish's organ system is very similar to that of humans, easier drug administration, because of the immature immune system development the xenografts are not rejected in 48-72 HPF embryos [71-73]. Apart from clinical advantages, zebrafish's availability and breeding is very easy, and maintenance is very low and economical. The maintenance cost of zebrafish is just 1% of the total maintenance that of mice [73].

Zebrafish have been used in drug screening in various cancer researches [74]. Similar to that of mice, genetic modification can also be made in zebrafish and transgenic zebrafish can be used as a model of several types of cancer. Zebrafish have already been used to make many transgenic models of diseases such as leukaemia, melanoma and pancreatic cancer. Zebrafish embryos are used to study the xenograft tumours, metastasis and angiogenesis [75, 76].

Caudal fin regeneration model is based on the regeneration property of zebrafish. Zebrafish can undergo angiogenesis very quickly, which is also a key factor in metastasis and tumour development [78, 79]. Angiogenesis growth factors released by tumour cells like VEGF and FDF are responsible for tumour development and the absence of these factors will let the tumour in a state of dormancy [79, 80]. Adult zebrafish (Danio rerio) is kept for an acclimatization period for about 15 days in laboratory conditions and are divided into experimental groups, i.e., Vehicle control group, Standard Group, and test groups. Caudal fins of zebrafish are amputated using a razorblade (50% lesion size), after which the regeneration of the caudal fin is photographically documented. Prior to the step of amputation of fins and during the observation, zebrafish are anesthetized using tricaine. Images are captured frequently to observe the growth of the caudal fin at every stage [79-82].

Following vascular parameters are evaluated: 1. Total Regenerated Area (TRA) 2.Vascular Projection Area (VPA)

CAM Model: The Chick Chorioallantoic Membrane (CAM) is an embryonic membrane which comprises of dense blood vessels and lymphatic vessels. CAM has a dense network of capillaries and is highly accessible as it is very close to the outer shell of the egg. Thus, the CAM model is anefficienttool to observe and track the angiogenesis progression [83-85]. Mainly there are two types of CAM assay, *in-ovo* method, and *ex-ovo* method. The *in-ovo* is the earliest method to be used which had the advantage of higher viability of the embryo but limited accessibility to the CAM. Later, *ex ovo* method was introduced in which the cultures of chick embryo were shell-less. This greatly enhanced the accessibility to the CAM, but the viability rate was affected. However, the viability rate could be enhanced using certain measures [83]. Certain modifications of these models are also available, like the cup-CAM method, which is economical and also provides higher accessibility and viability of embryo till the advanced embryonic developmental stages [83].

CAM model basically includes grafting of tumour inducing agents and test compounds onto the developing CAM as per the respective experimental groups [66]. The eggs used are of fertilized White Leghorn chicken eggs, which are 3 days old. The CAM is then prepared for the grafting by removing 3 ml of albumin out using a syringe to create an air sac. Air sac allows for intact CAM development. On day 8 or 9, a square window cut out is made on the shell of the egg. The grafts are placed gently over the CAM via a window cut-out made on the shell of the egg. The grafting material is of various types but mainly includes a small disk made up of Whatman paper, nitrocellulose membranes, gelatin sponges, inert synthetic polymers that are soaked in the test compounds or the tumour inducing agent. After the disk application, the window is sealed to prevent decontamination. Visual observation and quantification of the angiogenesis are performed 4 days after the disk application [66, 67, 83-85]. Following vascular parameters are evaluated: 1. Total Regenerated Area (TRA) 2. Vascular Projection Area (VPA) [66, 79-82].

CONCLUSION: Conventional preclinical screening models for anticancer drugs, although are powerful tools in drug development but are quite fallible and thus the situation of 95% attrition rates in clinical trials. Currently, drug development for cancer involves a limited number of screening models with respect to the preclinical stage. Xenograft model, hollow fiber assay are extensively used in vivo models but are limited in delivering high correlation because of the lack of immune system participation in the evaluation process. Models like GEMM overcome such limitations; however, GEMM requires the use of advanced DNA sequencing technologies like CRISPR for delivering the oncogene or knocking off a tumour suppressor gene. This not only increases overcall cost but also increases the time required for drug development. Most of the time, but not necessarily always, the assessment of outcomes in *in-vivo* techniques in general for anticancer drug screening is subjective and relies on personal interpretation, which can bring upon discrepancies. *In-vitro* techniques, however, make use of standardized evaluation of the outcome like spectrophotometric techniques, microtiter plate reader in HTS.

Non-conventional newer screening models like Zebrafish: Caudal fin regeneration assay and CAM Assay have been developed and are being continuously explored and modified to increase their reliability and efficiency in screening anticancer drugs. In the current scenario, conventional and newer screening models, if combined and used for validation of each other, would be most beneficial for determining anticancer activity.

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